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Full Length Research Paper

In vitro propagation and free radical studies of *Smilax zeylanica* Vent

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A method of micropropagation through multiple shoots formation from nodal segments of *Smilax zeylanica* Vent. has been developed. The nodal explants were cultured on half strength MS medium containing BA (0.5 mg/l) and IAA (1 mg/l) with activated charcoal (100 mg/100 ml) produced single shoot within 7-10 days. Shoots were multiplied by using nodal segments of *in vitro* regenerated shoot in modified half strength MS medium supplemented with KIN (2 mg/l), L-Glu (0.5 mg/l) and activated charcoal (100 mg/100 ml). Two shoots were formed. Rooting of the microshoots was achieved in half strength MS medium fortified with IBA (1 mg/l) within three weeks. The rooted plantlets transferred to potting medium containing vermiculite, sand and coir pith (1:1:1). Survival of the plantlets under *ex vitro* condition was 70%. *In vitro* antioxidant activities of leaf and stem extracts were performed. Among the extracts tested, ethanol extract of the stem showed maximum DPPH (52.361%) scavenging activity and chloroform extract of stem inhibited hydroxyl radical mediated linoleic acid oxidation up to 50.87%. Ethanol extract of leaf showed maximum reducing power of 0.53. The total free phenolics were found to be 293.3 µg in the ethanol extract of leaf.

Key words: ***Smilax zeylanica***, nodal culture, rooting, antioxidant activity.

INTRODUCTION

Smilax zeylanica Vent. commonly called “sarasaparilla”, is an evergreen woody climber endemic to Western Ghats of Southern India. It is a slow growing riparian species distributed up to 1200 m altitude. The roots are used to treat syphilis, gonorrhea, swellings, abscesses and boils (Nadkarni, 1976). Species of ***Smilax*** are gaining importance as a source of steroidal saponins isolated from the roots (Sautour et al., 2005). The plant number has declined presumably due to several biotic and abiotic factors. The major threat factor for seed is loss to insects (Mutharaian, 2003).

Conventional propagation via vegetative cutting is not practiced due to difficulty in rooting. Micropropagation has many advantages over conventional vegetative propagation and could be used to complement conservation and to utilize genetic diversity. In this paper, as a part of conservation effort, we describe a reliable method for large scale multiplication of ***S. zeylanica in vitro***, using nodal explants. In addition the antioxidant activity also studied. Recent studies showed that a number of plant products including polyphenolic substances and various plant extracts exert antioxidant actions. Natural antioxidants can be used in the food industry, and there is evidence that these substances may exert their antioxidant effects within the human body (Larsen, 1997). Almost all ***S. zeylanica***a parts are used, with the extract from leaves and stem being the most important source of antioxidants.

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Abbreviations: BA, 6-benzyl adenine; **KIN**, kinetin; **IAA**, indole-3- acetic acid; **IBA**, indole-3-butyric acid; **NAA**, naphthalene acetic acid; **L-GLU**, L-glutamine; **DPPH**, 1, 1-diphenyl-2-picrylhydrazyl; **TBA**, thiobarbituric acid; **MDA**, malonaldehyde.

MATERIALS AND METHOD

Tissue culture

Healthy green nodal segments were collected from the plants maintained in the Botanical Garden, Department of Botany, Bharathiar University, Coimbatore, Tamil nadu, India. The excised

nodal segments (3 cm long) were washed with running tap water followed by 5% (v/v) teepol treatment for 5 - 10 min and then treated with fungicide (Bavistin) for 20 min. The treated explants were washed with distilled water. Then the explants were pre-treated with antioxidants namely citric acid (0.15 mg/l) and ascorbic acid (0.1 mg/l) for 5 - 10 min to eliminate phenolic exudation (Sondhal and Sharp, 1977). Antioxidant pretreated explants were disinfected with 0.1% mercuric chloride for 3 min and finally rinsed with sterile distilled water under aseptic condition. The pH of the medium was adjusted to 5.8 before adding 0.8% (w/v) agar (Hi Media) and molten media (15 ml) were poured into 25 x 150 mm culture tubes (Borosil, Mumbai) and autoclaved at 121ºC and 1.06 kg cm^{-2} pressure for 20 min. The cultures were incubated at 25±2ºC under a 16 h photoperiod of 50 - 60 µ mol $m^{-2}S^{-1}$ flux density provided by cool white fluorescent tubes.

The nodal explants were cultured on two different media, half strength and modified half strength MS medium supplemented with various growth regulators such as BA (0.5 - 2 mg/l), KIN (2mg/l), IAA (0.5 - 1 mg/l), NAA (0.5 mg/l), and activated charcoal (100 mg/100 ml) was also added to the above media. To avoid the phenolic exudation the explants were pretreated with antioxidants such as citric acid (0.15 mg/l) and ascorbic acid (0.1 mg/l). The number of days taken for shoot induction, percentage frequency of shoot induction and number of shoots formed per explant were recorded. The nodal explants from the ***in vitro*** shoot were subcultured in the modified half strength MS medium supplemented with KIN (0.5 – 2 mg/l), L-Glu (0.5 - 1 mg/l) and activated charcoal (100 mg/100 ml). For the induction of roots, the microshoots were cultured on full strength and half strength MS basal medium supplemented with IBA (0.5 - 1 mg/l). The regenerated plantlets were washed with sterile distilled water and treated with fungicide (5% Bavistin) and transferred in to pot containing sterilized vermiculite sand and coir pith in the ratio of 1:1:1 and sprayed with liquid MS medium. Survival rate was noted three weeks after transferring to the pots.

Preparation of plant extracts

To study the antioxidant activity the fresh leaves were shade dried and powdered. The leaf and stem powder (50 g) was extracted separately with 100 mL of chloroform, ethyl acetate and ethanol using Soxhlet apparatus until the solvent became colorless. The extracts were filtered and evaporated to dryness by a rotary evaporator at 30ºC. The extracts were dissolved in dimethyl sulfoxide at a concentration 50 mg/5 ml, from this 500 µL was taken and the following assays were performed.

Determination of amount of total phenolic compounds

The amount of phenolic content of extracts was determined by the method described by Singleton et al. (1999). 500 µl of extract (contains 500 µg of extract) was transferred to a 100 mL Erlenmeyer flask and the final volume was adjusted to 46 mL by addition of distilled water. Folin-Ciocalteau reactive solution was added and incubated at room temperature for 3 min. 2% Sodium carbonate solution was added and the mixture was shaken on a shaker for 2 h at room temperature. The absorbance was measured at 760 nm. Gallic acid was used as the standard for a calibration curve. The phenolic compound content was expressed as gallic acid equivalent.

DPPH radical scavenging activity

Scavenging activity on DPPH free radicals by the extracts were assessed according to Blois (1958) with a slight modification. 500 µL of the extract solution was mixed with 1 mL of 0.1 mM DPPH in ethanol solution and 450 µL of 50 mM Tris-HCl buffer (pH 7.4) was added. The solution was incubated at 37ºC for 30 min and reduction of DPPH free radicals was measured by reading the absorbance at 517 nm. Control was maintained. Ascorbic acid solution was used for comparison. This activity is given as % DPPH scavenging and calculated according to the following equation:

% DPPH scavenging = [(control OD – sample OD) / (control OD)] X 100

Reducing power

500 µL of extract was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The reaction mixture was incubated at 50ºC for 30 min. Afterwards, 2.5 mL of 10% trichloroacetic acid was added to the above mixture and centrifuged for 10 min at 3000 rpm. 2.5 mL of supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Absorbance was measured at 700 nm. Ascorbic acid solution was used for comparison (Yildrim et al**.,** 2001).

Antioxidant activity

The antioxidant activity was measured through ammonium thiocyanate assay (Lee et al., 2002). 500 µL of the extract, 200 µL of diluted linoleic acid (25 mg/mL 99 ethanol) and 400 µL of 50 mM phosphate buffer (pH 7.4) was mixed and incubated at 40°C for 15 min. 100 µL aliquot from the reaction mixture was mixed with reaction solution containing 3 mL of 70% ethanol, 100 µL of ammonium thiocyanate (300 mg/mL distilled water) and 100 µL of ferrous chloride (2.45 mg/mL in 3.5% hydrochloric acid). Final reaction solution was mixed and incubated at room temperature for 3 min. Absorbance was measured at 500 nm. Linoleic acid emulsion without extract served as control. Inhibition of linoleic acid oxidation was calculated by using the following formula:

% inhibition = [(control OD – sample OD)/control OD)] X 100.

RESULTS AND DISCUSSION

The technique of ***in vitro*** propagation has been highly successful with herbaceous species, but most of the woody species responded poorly because of low rate of shoot proliferation, poor growth and phenolic exudation (Linington, 1991). The same growth response was also observed in ***S. zeylanica*** a woody climber. Exudation and browning of the explants were overcome when the explants were pretreated with antioxidants such as citric acid and ascorbic acid (0.15 and 0.1 mg/l, respectively). As shown in Table 1 the pretreated nodal explants responded on half strength MS medium fortified with BA (0.5 mg/l), IAA (1 mg/l) and activated charcoal (100 mg/100 ml) for early shoot induction. Percentage of shooting was 100 and shoot length was 9 cm after 45 days of culture (Figure 1a).

Shoot tip necrosis was observed during the regeneration of shoots in ***S. zeylanica***. Several explanations have been given by various workers for the occurrence of shoot tip necrosis. Exudation of phenolics has been reported to cause necrosis of shoot tips in ***in vitro*** culture

Table 1. Effect of various hormonal concentrations on the shoot initiation from the nodal explants of ***S. zeylanica*** on half strength MS medium.

Hormonal concentration (mg/l)					Number of days taken for shoot initiation	Frequency of forming explant (%)	Average length of shoot (cm)
BA	KIN	IAA	NAA	AC			
0.5	-	-	-		10	70	5
1	-	-	-		7	70	5
0.5	-	1	-		7	100	9
1	-	0.5	-		10	60	8
1	-	1	-	100	10	70	5.7
2	-	1	-		10	60	8
0.5	-	-	0.5		7	60	3.8
1	-	-	0.5		7	60	3.6
2	-	-	0.5		7	60	2.2
-	2	-	-		8	80	6.0

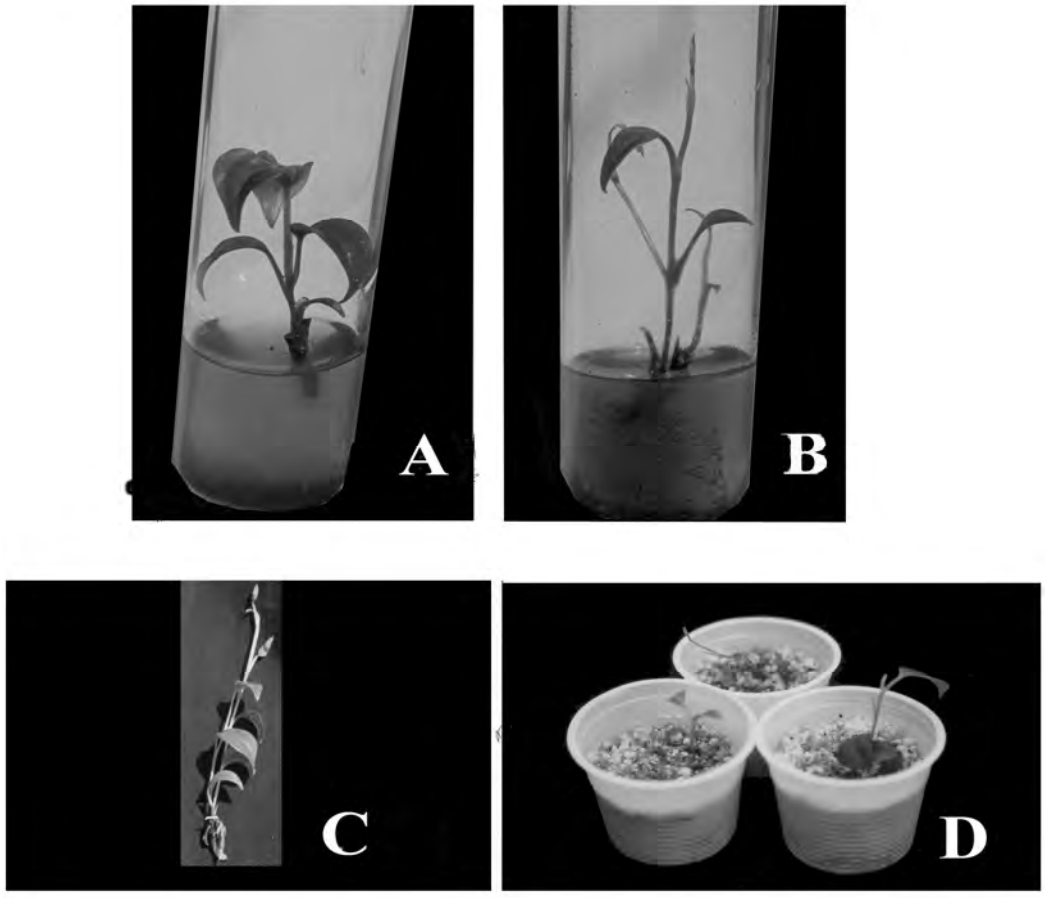

Figure 1. A. Regenerated shoot with leaves in half strength MS+BA (0.5 mg/l) + IAA (1 mg/l) + AC (100mg/100 ml). **B.** Regeneration of shoots from ***in vitro*** nodal explants in modified half strength MS + KIN (2 mg/l) + L-glutamine (0.5 mg/l) after first subculture. **C.** Formation of roots on half strength MS + IBA (1mg/l). **D.** ***In vitro*** plantlet hardened in the potting medium (Vermiculite: Sand: Coirpith) in the ratio of 1:1:1.

of many tree species (Standardi and Romani, 1990; Bellarosa, 1988). In many plant species, shoot tip necrosis is consider to be a physiological disorder associated with rooting. According to Katacva et al**.** (1991), the cultivation of apical microshoots for several months in case of apple varieties with low rooting ability on a cytokinins free

Table 2. Effect of modified half strength MS medium + activated charcoal (100mg/100ml) and various concentrations of glutamine and kinetin on multiple shoot induction after subculture.

KIN (mg/l)	GLU (mg/l)	Number of days taken for shoot initiation	Frequency of shoot forming explant (%)	Number of shoots per explant	Average length of shoots
0.5	0.5	12	60%	1	6
1	0.5	11	40%	1	5.3
2	0.5	14	60%	2	2.9±1.8
1	1	16	60%	1	7

Table 3. Effect of IBA on rooting of the micro shoots.

Medium	Concentration (mg/l)	Response	Number of roots per explant	Length of roots (cm)
MS	0.5	No response	-	-
MS	1	No response	-	-
1/2MS	0.5	Root formation	2.8	1.44 ± 0.12
1/2MS	1	Root formation	6.6	3.32 ± 0.89

medium lead to cytokinins exhaustion from the shoots. Without roots the shoots are devoid of a source of endogenous cytokinins; cytokinins deficiency leads to the cessation of cell division in the apical meristem and to cellular necrosis. In ***S. zeylanica***, shoot tip necrosis was controlled by modifying the concentration of $CaCl_2$ and $MgSO_4.7H_2O$ in the medium. On the contrary in ***Butea*** necrosis was prevented without elimination or increasing calcium and sulphur in MS basal medium (Kavitha Kulkarni et al**.,** 2000).

The ***in vitro*** shoot developed was subcultured in modified half strength MS medium. A combination of KIN (2 mg/l), L-Glu (0.5 mg/l) and activated charcoal (100 mg/ 100 ml) produced two shoots from the ***in vitro*** nodes after two weeks of culture (Table 2). The percentage frequency of shoot formation was 60. Average length of shoots was recorded as 2.9±1.8 (Figure 1b). Glutamine is frequently employed in the culture medium as an organic nitrogen source (Franklin and Dixon, 1994). Many papers have shown that the use of exogenous glutamine can be beneficial for ***in vitro*** culture, increasing the regeneration rate and biomass of the explants (Franklin et al., 1991; Shetty et al., 1992; Ogita et al**.,** 2001; Sudarsana Rao et al**.**, 2001; Vasudevan et al., 2004). Addition of glutamine with kinetin was found to be necessary for induction of more than one shoot, whereas glutamine in combination with BA did not favor the development of shoot.

Table 3 shows the rooting ability of the isolated shoot in ***S. zeylanica*** in half strength MS basal medium supplemented with IBA (1 mg/l) was found to be most effective to achieve rhizogenesis of microshoots. Within three weeks, 6.6 mean numbers of shoots were formed and the length of the roots were measured to be 3.32±0.89 cm (Figure 1c). It is well documented in many woody plants that modifications such as the reduction in the salt concentrations (Wang, 1978), and addition of auxins (George and Sherrington, 1984) can promote root development, which in agreement with ***S. zeylanica***, where half strength MS medium supplemented with auxins facilitated root growth.

The ***in vitro*** developed plantlets were washed thoroughly with sterile distilled water and transferred to the potting medium containing vermiculite, sand and coir pith in the ratio of 1:1:1 (Figure 1d). Initially MS basal liquid medium (without sucrose) was added to the above potting medium. Nearly 70% of the plantlets survived. The protocol reported here could be used for micropropagation of this woody climber, which is threatened and restricted in its distribution.

Since ***Smilax*** is reported to be rich in phenolic compounds the antioxidant assays were performed. Phenolic compounds are called high level antioxidants because of their ability to scavenge free radical and reactive oxygen species such as singlet oxygen, superoxide free radicals and hydroxyl radicals. The antiradical activity of flavornoids and phenolics is principally based on the redox properties of their hydroxy groups and the structural relationships between different parts of their chemical structure (Burda et al**.,** 2001; Rice-Evans et al**.,** 1996, 1997). The present study revealed the presence of total free phenolics in the ethanol extract of leaf and stem (293.3) and (236.3) µg respectively (Table 4).

Yildrim et al. (2001) suggested that there may be relationship between phenolic compounds and reducing power. The condition was well observed in ethanol extract of leaf which has greater amount of phenolic compounds showed maximum reducing power of 0.534. So it can be suggested that phenolics may be responsible for reducing power of the ethanol extract (Table 5). DPPH is a nitrogen-centered free radical. Antioxidants

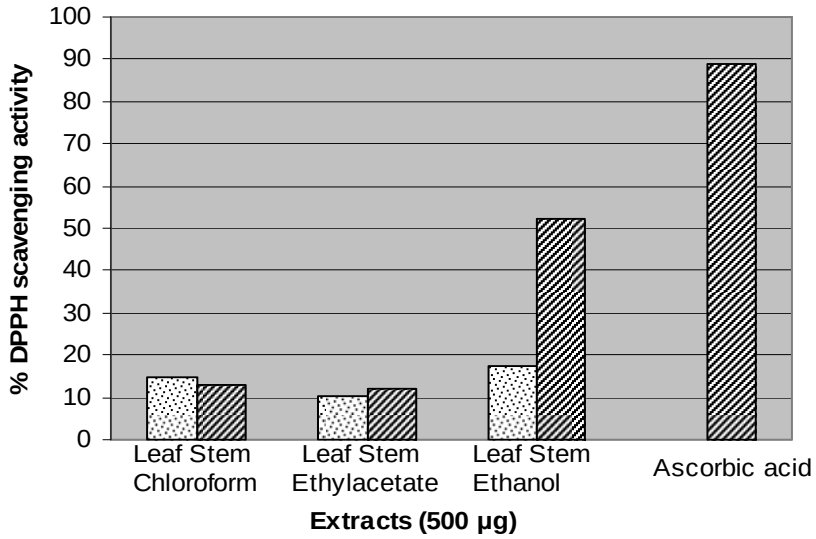

Figure 2. DPPH radical scavenging activity of ***S. zeylanica*** leaf and stem extracts.

Table 4. Amount of total phenolic compounds in ***S. zeylanica*** leaf and stem extracts.

Extracts[a]	Gallic acid equivalent (µg)	
	Leaf	Stem
Chloroform	21.6 ± 0.55[b]	18.6 ± 1.1
Ethyl acetate	18 ± 1	23.6 ± 0.5
Ethanol	293.3 ± 3.0	236.3 ± 2.0

[a]500 µg of extract was used for phenolic compound determination.
[b]Values are mean ± standard deviation; n= 3 samples.

Table 5. Reducing power of the leaf and stem extract of ***S. zeylanica***.

Extracts[a]	Absorbance (700 nm)	
	Leaf	Stem
Chloroform	0.174 ± 0.0020	0.135 ± 0.002
Ethyl acetate	0.163 ± 0.003	0.186 ± 0.001
Ethanol	0.534 ± 0.001	0.436 ± 0.001
Control	0.039 ± 0.0005[b]	
Ascorbic acid	0 3.639 ± 0.0005	

[a]500 µg of extract was used. Values are mean ± standard deviation; n= 3 samples.
[b]In control there was no extract. High absorbance indicates high reducing power.

react with DPPH and convert it to α,α-diphenyl, β-picrylhydrazine. The degree of discoloration indicates the scavenging potentials of antioxidants. Activity of extracts is attributed to their hydrogen donating ability. The present study showed that the ethanol extract of stem is a potent scavenger of nitrogen centered free radical up to 52.361% in comparison to the 89% shown by ascorbic acid used as standard (Figure 2). Ethanol extract is rich in phenolic compounds; it might be the responsible for the observed DPPH radical scavenging activity. Methanol extract of ***Smilax china*** root showed relatively high DPPH radical scavenging activity, with an average IC50 value of 7.4 mg/ml.

Scavenging activity of leaf and stem extracts on hydroxyl generated in a Fe^{3+} dependent manner was studied by ammonium thiocyanate assay. Chloroform extract of stem inhibited linoleic acid oxidation of 50.87% which was the maximum activity observed, followed by ethanol extracts of leaf and stem which showed 41.14 and 40.15%, respectively (Figure 3). Reaction solution generated hydroxyl radicals oxidize linoleic acid resulting in peroxide formation. Peroxide oxidizes Fe^{2+} to Fe^{3+} and then this ferric ion reacts with SCN^- from the $FeSCN^{2+}$ complex, which has maximum absorbance at 500 nm. In presence of antioxidants, color intensity is weak (Yildrim et al., 2001). To decrease hydroxyl radical generation, antioxidants chelate metal ions or it should directly scavenge hydroxyl radicals. DPPH radical scavenging activity, reducing powers and hydroxyl mediated linoleic acid oxidation of all the extracts was lower than the ascorbic acid, a known antioxidant.

Abstraction of a hydrogen atom from the double bond in the fatty acid is considered as an initial step in the sequence of peroxidation of membrane or polyunsaturated fatty acid. The free radicals tend to be stabilized by a molecular rearrangement to produce a conjugated diene, which then easily react with oxygen molecule to give peroxy radical. Peroxy radicals can abstract hydrogen atom from another molecule to give a lipid

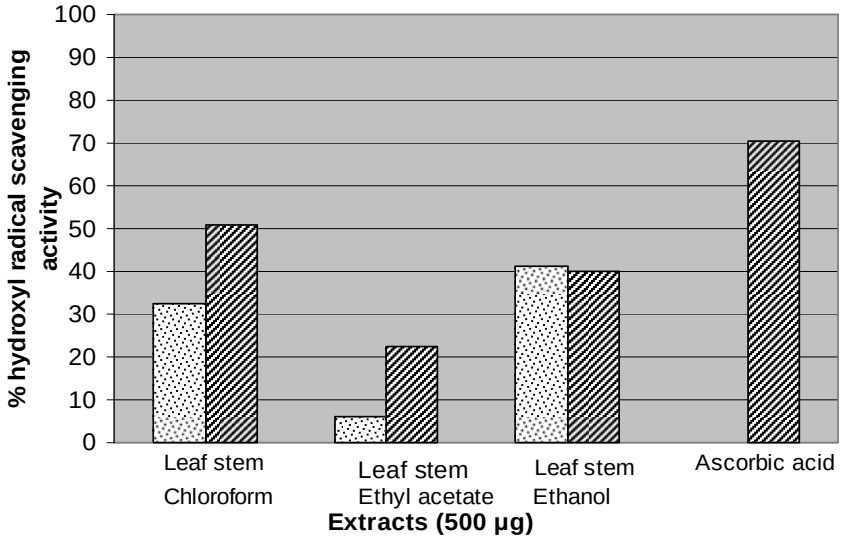

Figure 3. Inhibitory effect of hydroxyl radical mediated linoleic acid oxidation of leaf and stem extracts of ***S. zeylanica.***

hydroperoxide. This cyclic peroxides fragment to aldehydes including malonaldehyde (MDA) polymerization products. MDA is the major lipid peroxidation product and is used to study the lipid peroxidation in rat liver homogenate. Determination of lipid peroxide content is carried out indirectly by means of derivatizing malonaldehyde with thiobarbituric acid (TBA) at high temperature and acidic conditions MDA is a very reactive species and takes part in cross-linking of DNA with potentials and also damaging liver cells.

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